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Genetic Diversity Estimates for the Genus *Hydrangea* and Development of a Molecular Key Based on SSR

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Abstract. **Using 14 codominant microsatellite markers that amplify loci across 14 different *Hydrangea* L. species, we analyzed gene diversity and genetic similarity within *Hydrangea*. Samples also included *Dichroa* Lour., *Platycrater* Sieb. and Zucc., and *Schizophragma* Sieb. and Zucc. genera to establish their relatedness to *Hydrangea* species since previous work suggests they may be closely related. Our results support the close affiliation between *Macrophyllae* E.M. McClint. and *Petalanthe* (Maxim.) Rehder subsections and their separation from the other *Hydrangea* species. Most of the *Hydrangea* species analyzed cluster within their designated sections and subsections; however, genetic distance between species within each subsection varied considerably. Our data suggest that morphological analyses which labeled *H. serrata* (Thunb.) Ser. as a subspecies of *H. macrophylla* (Thunb. Ex J.A. Murr.) Ser. are probably more accurate than recent genome size data suggesting *H. macrophylla* ssp. *macrophylla* (Thunb.) Ser. and *H. macrophylla* ssp. *serrata* (Thunb.) Makino are separate species. Gene diversity estimates indicate that 64.7% of the total diversity is due to differences between species and 49.7% of the overall variation is due to differences between subsections. Low diversity suggests a lack of gene flow between species and subsections and underscores the difficulty in making wide hybrids. Since only 35.3% of the genetic variation is common to all species, unique alleles were used to develop a molecular key for unambiguous species identification and interspecific hybrid verification. Genetic similarity estimates for all 85 samples suggests a roadmap for introgressing horticulturally important traits from different *Hydrangea* species.**

Hydrangeas are the fourth top selling deciduous flowering shrub in the United States with annual sales in excess of $32 million. *Hydrangea macrophylla* is the most popular species, but four other members of the genus are cultivated in this country. While most of the *H. macrophylla* cultivars available today were developed in Europe during the first half of the 20th century, there are currently breeding efforts under way targeted at developing reliable, low-maintenance *H. macrophylla* with a wide array of desirable ornamental traits. Breeding efforts are also under way to improve *H. quercifolia* Bartr., *H. paniculata* Sieb., and *H. arborescens* L. Despite a recent increase in hydrangea breeding and interest in germplasm conservation, relatively little is known about the relatedness between *Hydrangea* species. The last major revision of *Hydrangea* taxonomy was carried out in 1957 by McClintock, who established the species designations commonly used today.

In an effort to accelerate breeding of new cultivars, we established simple sequence repeat (SSR) markers for *Hydrangea*. During development of a marker-assisted breeding program for *H. macrophylla*, we discovered 14 microsatellite loci that amplify across *Hydrangea* species. Here we used these SSR loci to better understand the relationships within *Hydrangea* species and between *Hydrangea* and related genera that may represent alternative sources of genetic diversity for hydrangea breeding programs (Fan and Xiang, 2003).

Hydrangeoideae is a subfamily of the Hydrangeaceae and includes two tribes, Cardiandreae with two genera and Hydrangeae with nine genera including at least six horticulturally important *Hydrangea* species. While the tribe *Hydrangeae* is considered monophyletic, seven of the genera in *Hydrangeae* are proposed to be polyphyletic including *Broussaisia* Gaudich., *Decumaria* L., *Dichroa*, *Hydrangea*, *Pileostegia* Hook. f., and Thomson, *Platycrater*, and *Schizophragma*. Two other genera, *Cardiandra* Sieb. and Zucc. and *Deinanthe* Maxim., are considered basal. Phylogenetic work using DNA sequence data from rbcL and matK loci and morphological characters supports four clades within the polyphyletic group (Fig. 1) but relationships between other species remain unresolved (Hufford, 1995, 1997, 2001; Hufford et al., 2001).

McClintock (1957) successfully organized *Hydrangea* into 23 species, largely by avoiding pubescence characters and carefully mapping their disjunct geographic distributions. She developed a morphological key to identify species and divided the genus into two sections, *Hydrangea* Maxim. and *Cornidia* (Ruiz and Pavon) Engler, which contain six and two subsections respectively (Table 1). Her taxonomic designations are generally supported by recent

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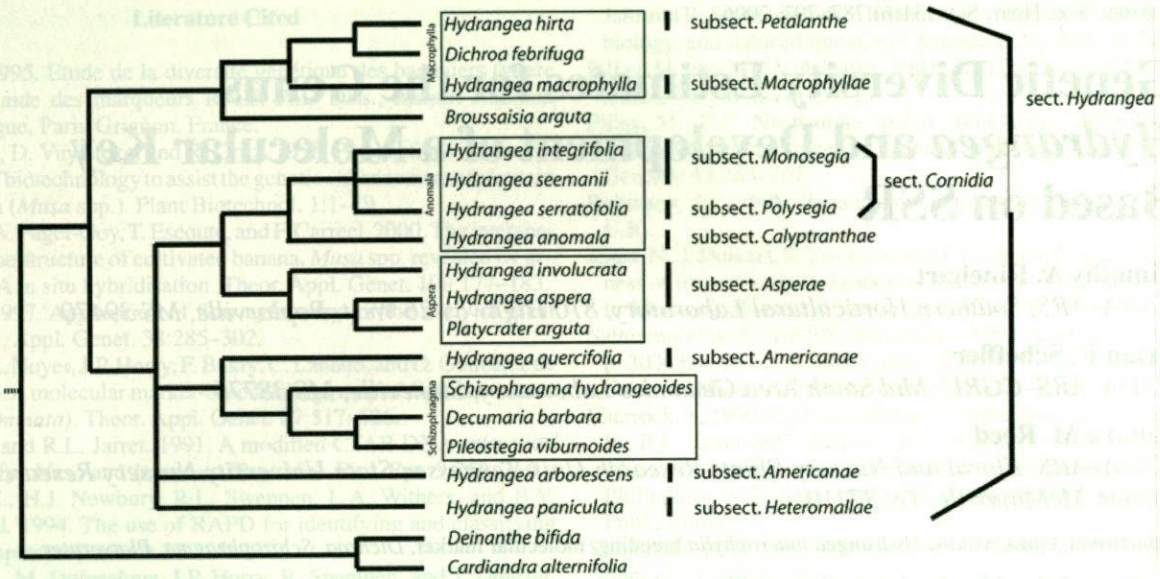

Fig. 1. Relationships between Hydrangea and other genera are shown in a strict consensus cladogram for Hydrangeaceae modified from Hufford, 2001 which included DNA sequence data from rBCL genes. Clades are boxed. Sections (sect.) and subsections (subsect.) are shown to the right of the dendogram.

research and are accepted as the current standard (Hufford, 2001). However, there are several controversies that can be addressed with the SSR data presented here.

First, the taxonomic treatment of *Hydrangea* subsection *Macrophyllae* has long been disputed. *Hydrangea macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* were initially considered to be closely related species (Wilson, 1923), but were combined into a single species by Makino (Hara, 1955). Haworth-Booth (1984) recommended dividing the subsection into four species. He believed that *H. macrophylla* was a complex hybrid (×*H. macrophylla*) produced from hybridizations of a coastal species (*H. maritima* Sieb. and Zucc.), with three woodland species (*H. acuminata* Sieb. and Zucc., *H. japonica* Sieb. and Zucc. and *H. thunbergii* Sieb.). Because the traits that Haworth-Booth ascribed to the four species were primarily ecological and cultural responses, McClintock (1957) did not support his reclassification of the subsection or a hybrid origin for *H. macrophylla*. While she kept *serrata* at the subspecies level, recent publications by several leading authorities on *Hydrangea* have reverted back to the species designation (Dirr, 2004; Mallet, 1994; Lawson-Hall and Rothera, 1995; van Gelderen and van Gelderen, 2004). Most of the argument supporting separation of *H. macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* is based on morphological and geographical distribution differences between the two groups; however, molecular data has recently been presented in support of elevation of *serrata* to species status. Flow cytometric measurements of propidium iodide stained nuclei revealed that, although both sets of plants had the same chromosome number, the mean nuclear DNA content of 16 *macrophylla* cultivars was 5.8% greater than that of 18 *serrata* cultivars (Zonneveld, 2004). Although DNA content has limited phylogenetic usefulness (Cerbah et al., 2001), it has been used to characterize species, verify triploid cultivars, and uncover interspecific hybrids within *Hydrangea* (Demilly et al., 2000; Zonneveld, 2004).

Second, the genetic distance between *Macrophyllae* and *Petalanthe* and the other subsections has not been determined, nor has it been resolved for the four other subsections that originate in eastern Asia. The relationship between *H. quercifolia* and *H. arborescens* in subsection *Americanae* (Maxim.) Engler is also relatively unresolved since they appear distantly related despite being native to overlapping geographic regions.

Finally, relationships between various species of *Hydrangea* and members of related genera have been proposed but are not fully understood. *Dichroa* is supposedly related to *H. macrophylla* but its relationship within *Macrophyllae* is not well defined (Hufford, 2001). *Schizophragma* is sometimes allied with subsection *Americanae* and sometimes subsection *Heteromallae* Rehder (Hufford, 2001; Hufford et al., 2001). *Platycrater* is thought to be similar to subsection *Asperae* Rehder.

In addition to clarifying relationships between species, gene diversity estimates and genetic similarity clustering will be important information for choosing parents for wide hybridization studies. There has been difficulty in creating wide hybrids in *Hydrangea*, which may be due in part to differences in genome size and chromosome number between parents. *Hydrangea arborescens*, which has a small genome [2.64 pg DNA per diploid nucleus (2C)] and 2n = 38 chromosomes, has been successfully crossed with *Hydrangea involucrata* Sieb., which has fewer chromosomes (2n = 30) but a larger genome (5.36 pg/2C) (Cerbah et al., 2001; Jones and Reed, 2006). Embryo rescue was used to produce hybrids between *H. macrophylla* and *H. quercifolia*, *H. arborescens* and *H. paniculata*, but all three hybrids were weak, aneuploid and/or sterile (Kudo et al., 2002; Kudo and Niimi, 1999; Reed, 2004; Reed et al., 2001). *Hydrangea quercifolia* has the smallest known genome size (2.17 pg/2c) and has 2n = 36 chromosomes (Cerbah et al., 2001; Zonneveld, 2004). The genome size for *H. paniculata* is the largest of the *Hydrangea* species examined at 7.00 pg/2C (Cerbah et al., 2001; Zonneveld, 2004), but it is a tetraploid with 2n = 72 chromosomes (Sax, 1931). The only *Hydrangea* interspecific hybrid to be introduced into the trade is a hybrid between *H. aspera* D. Don and *H. involucrata* (Dirr, 2004), which are in the same subsection.

Genome size does not necessarily coordinate with chromosome number or fertility. Cerbah estimates 2n = 36 for *H. macrophylla* cultivars but some triploid cultivars have been identified (Cerbah et al., 2001; Demilly et al., 2000; Zonneveld, 2004). Interestingly, both ploidy types appear fertile (K. Jones, personal communication). *Hydrangea macrophylla* ssp. *serrata* has 2n = 36

Table 1. Overview of the *Hydrangea* genus including infrageneric divisions, geographic origin, and common synonyms for taxa.

Section	Subsection	Species	Subspecies	Native habitat	Possible synonyms
Hydrangea Maxim.	*Americanae* (Maxim.) Engler	*H. arborescens* L.	*arborescens* L.[z]	Eastern U.S.	
			radiata (Walter) E.M. McClint.[z]		
			discolor (Ser. ex DC.) E.M. McClint.[z]		
		H. quercifolia W. Bartram[z]		Eastern U.S.	
	Asperae Rehder	*H. sikokiana* Maxim.[z]		Eastern Asia	
		H. involucrata Sieb.[z]		Eastern Asia	
		H. aspera Buch.-Ham. ex D. Don	*aspera* Buch.-Ham. ex D. Don[z]	Eastern Asia	
			strigosa (Rehder) E.M. McClint.[z]		
			robusta (Hook. f. and Thomson) E.M. McClint.[z]		
			sargentiana (Rehder) E.M. McClint.[z]		*H. sargentiana*
			villosa Rehder[z]		*H. villosa*
	Calyptranthae (Maxim.) E.M. McClint.	*H. anomala* D. Don	*anomala* D. Don[z]	Eastern Asia	
			petiolaris (Sieb. and Zucc.) E.M. McClint.[z]		*H. petiolaris*, *H. quelpartensis*
	Petalanthe (Maxim.) Rehder	*H. hirta* (Thunb.) Sieb.		Eastern Asia	
		H. scandens (L. f.) Ser.	*scandens* (L. f.) Ser.	Eastern Asia	
			liukiuensis (Nakai) E.M. McClint.[z]		*H. luteovenosa* *H. angustipetala*, *H. lobbii*
			chinensis (Maxim.) E.M. McClint.[z]		
			kwangtungensis Merr.		
	Heteromallae Rehder	*H. paniculata* Sieb.[z]		Eastern Asia	
		H. heteromalla D. Don[z]		Eastern Asia	*H. xanthoneura*
	Macrophyllae E.M. McClint.	*H. macrophylla* (Thunb.) Ser.	*macrophylla* (Thunb.) Ser.[z]	Eastern Asia	
			serrata (Thunb.) Makino[z]		*H. serrata*
			stylosa (Hook. f. and Thomson) E.M. McClint.		
			chungii (Rehder) E.M. McClint.		
Cornidia (Ruiz and Pavon) Engler	*Monosegia* Briq.	*H. seemannii* Riley[z]		Mexico	
		H. asterolasia Diels		Central and South America	
		H. integrifolia Hayata[z]		Phillipines and Formosa	
		H. oerstedii Briq.		Central and South America	
		H. peruviana Moric. ex Ser.		Central and South America	
		H. diplostermona Standl.		Central America	
		H. preslii Briq.		Central and South America	
		H. steyermarkii Standl.		Central America	
	Polysegia Briq.	*H. serratifolia* (Hook. and Arn.) F. Phil.[z]		South America	
		H. tarapotensis Briq.		South America	
		H. jelskii Zahlbr.		South America	
		H. mathewsii Briq.		South America	

[z]Indicates taxa that were included in this study.

chromosomes, the same number as diploid *H. macrophylla* ssp. *macrophylla*, despite the fact that the average genome size for *H. macrophylla* ssp. *serrata* is 4.29 pg/2C, slightly less than 4.54 pg/2C estimated for *H. macrophylla* ssp. *macrophylla* (Zonneveld, 2004). Additionally, chromosome counts indicate 2n = 36 for *H. aspera* ssp. *aspera* Buch.-Ham. ex D. Don but 2n = 34 for *H. aspera* ssp. *robusta* (Hook. f. and Thompson) E.M. McClint. and *H. aspera* ssp. *sargentiana* (Rehder) E.M. McClint. Genome sizes for these taxa are 3.5, 3.5, and 3.36 pg/2C, respectively (Zonneveld, 2004).

The objective of this research was to use SSR markers to study relationships within *Hydrangea* and between *Hydrangea* and related genera. Codominant locus-specific markers such as SSR should provide relatively unbiased genetic distance estimates within and between subsections. Data presented here demonstrate the usefulness of SSR loci for species identification, germplasm conservation, and offer insight into future hydrangea breeding, particularly the creation of wide hybrids were relatively little progress has been made.

Materials and Methods

SSR DEVELOPMENT. Four SSR-enriched libraries, each containing ≈15,000 recombinant cells, were made from genomic DNA of *H. macrophylla* and *H. paniculata* (Genetic Information Services, Chatsworth, Calif.). From these libraries, 1152 random clones were sequenced and analyzed and 670 potential SSR primer pairs with an average repeat number of 11 were identified. Of these, 288 primer pairs were tested against a panel of 12 DNAs representing eight *Hydrangea* species. Fourteen loci produced polymorphic data for all samples tested suggesting they would amplify in all species within the genus (Table 1).

SAMPLING STRATEGY. Wildtype plants are not readily available for taxa listed in Table 1, particularly *H. macrophylla*, which have been subjected to prolonged cultivation. Tissue samples were collected from multiple sources for most taxa to guard against plant mislabeling. However, *Platycrater arguta* Sieb. et Zucc. and *Hydrangea sikokiana* Maxim. are each represented by only a single sample. As many cultivars as possible were sampled for each species resulting in uneven numbers of samples between species. The popularity of *H. macrophylla* was offset by choosing cultivars from different breeding programs to cover as much of the diversity as possible. We also included several remontant *H. macrophylla* cultivars that are economically important since our emphasis is on hydrangea breeding. For the sake of clarity, *H. macrophylla* ssp. *macrophylla* cultivars are listed as *H. macrophylla* and *H. macrophylla* ssp. *serrata* cultivars are listed as *H. serrata* in figures. The two other *H. macrophylla* subspecies, ssp. *stylosa* (Hook. f. and Thomson) E.M. McClint. and ssp. *chungii* (Rehder) E.M. McClint., are not represented in this study. Uneven sample sizes and deviations from Hardy–Weinburg equilibrium due to selection did not alter phenetic comparisons, which are based on genetic distance estimates.

SAMPLE PROCESSING. DNA was extracted from 1 × 1-cm pieces of fresh leaf tissue using Qiagen Plant Mini Kit (Qiagen, Valencia, Calif.), quantified using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, Del.), and diluted to a final concentration of 5 ng·μL^{-1}. Amplification was performed using a 3-primer protocol modified from (Waldbieser et al., 2003). Oligonucleotides corresponding to the forward primers listed in Table 2 were synthesized with 18 nucleotides added to the 5′ end (5′ CAGTTTTCCCAGTCACGA 3′) and were included in PCR reactions at final concentrations of 0.4 μM. Reverse primers included the addition of 5′ GTTT 3′ at the 5′ end and were included in PCR reactions at final concentrations of 1.8 μM. Reaction volumes were 10 μL and included 30 ng of genomic DNA along with 0.3 μL Advantage 2 polymerase mix (Clontech, Mountain View, Calif.), 0.2 μL Ultrapure dNTPs (Clontech), 1.0 μL Advantage2 PCR buffer (Clontech) and a third primer (5′ CAGTTTTCCCAGTCACGAC 3′) fluorescently labeled with FAM at a final concentration of 0.75 μM. PCR conditions included 3 minutes at 95 °C, 2 cycles of 95 °C for 1 min then 60 °C for 1 min, then 27 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 30 s. Cycling was finished after 68 °C soak for 4 min followed by storage at 4 °C. All cycling was carried out in 96-well plates using a Tetrad thermocycler (Bio-Rad Laboratories, Waltham, Mass.). Fluorescence-labeled PCR fragments were visualized by automated capillary gel electrophoresis on an ABI3100-Avant or

Table 2. SSR loci that were selected from a Hydrangea DNA library. Locus names correspond to SSR-enriched clones. Expected allele sizes were calculated based on the *H. macrophylla* DNA sequences which can be found by querying the National Institutes of Health genetic sequence database (GenBank) using the accession number listed.

Locus	GenBank accession no.	Repeat motif	Expected allele size (bp)	Actual allele size range (bp)	Forward primer	Reverse primer
STAB045_046	DQ521440	(TCA)8	151	153-174	AGAGGTCAGGCCTTGGAAAGATAC	AGAGGTCAGGCCTTGGAAAGATAC
STAB061_062	DQ521439	(CAC)4	96	96-119	CGGATCCAAAACCCTAATACAACA	ATAATGGAGGAGACGGAGAGTGTG
STAB111_112	DQ521451	(TCG)6	160	154-190	CTTCTTCCTCTTCTTTGGTGGTTG	AGAGAATGGAGATGACGACGATG
STAB125_126	DQ521450	(CTT)4	136	154-181	CAGTATCTCTGCCCAATCGAGAAT	TGACCAGAACGATGAGAATGAAAA
STAB157_158	DQ521449	(GCA)10	150	149-179	TCCATCGAGTTCAACTTCTTCTCC	AGTCGCAGATCTCACTTATTTCGG
STAB285_286	DQ521448	(CTG)8	159	159-183	CAGCCACCACTGCTACTGCTACTA	GATCCACCATTTTAGTGATTCGGA
STAB307_308	DQ521447	(TGG)4	109	108-132	GGGTTTATGGGCAGATGAATTTT	AAATTACCAATTTGCCCCATCTG
STAB309_310	DQ521447	(GCC)4	104	116-126	GGGCAAAATGGTAACCTTCCTATG	TGAAAAGTAATGCCTACCGATGCT
STAB311_312	DQ521446	(CCA)5	141	116-187	AGTGCCAGCATCACCACTAACATA	AACTATGGAAGTGGAGGCGGTTAT
STAB321_322	DQ521444	(TCT)7	159	164-185	CTAACAATTTCACCCATTTGAGGC	ATTAGGACTTACAGTCGCCGAGC
STAB391_392	DQ521443	(TCA)7	145	158-176	CCAACCCTTTCCTAAACTGCTCTT	AAGGGTGTGTTTGAGGATGTTGAT
STAB429_430	DQ521442	(CTG)6	83	84-103	GCTGGGATTGATTGTTGTACCC	ATGAGTAGCAGCAGAGGAGGAAGA
STAB457_458	DQ521441	(TTC)4	160	166-181	CAGGTGATGGAGATGGGGATATAG	TTGCAGTTGGAACTGATCAGAGAG
STAB501_502	DQ521445	(CAA)4	118	115-194	CATTTTGGTGGGTGGTTTAGGATA	TGTTGTTGCTGCTGTATTTGTGAA

Table 3. Source and cultivar information for Hydrangea, Dichroa, Platycrater and Schizophragma samples used in this study. Group designations were used to indicate plants labeled with synonyms. Accession numbers correspond to individual plants in research and display collections.

Taxa	Source[z] (accession no.)
H. arborescens 'Annabelle'	S. Reed (G156A)
H. arborescens ssp. *discolor* 'Frosty'	S. Reed (G665A)
H. arborescens ssp. *radiata* 'WhiteDome'	S. Reed (G671A)
H. quercifolia 'Alice'	Bell Family Nursery
H. quercifolia 'Pee Wee'	S. Reed (G688C)
H. quercifolia 'Semmes Select'	MAST (54603)
H. quercifolia 'Snowflake'	MAST (40201)
H. quercifolia 'SnowQueen'	Bell Family Nursery
H. sikokiana	M. Dirr
H. involucrata 1	MAST (45500)
H. involucrata 2	S. Reed (G331A)
H. involucrata 'Plena'	MAST (3405)
H. involucrata 'Tama Azisai'	Bell Family Nursery
H. aspera	S. Reed (G628A)
H. aspera 'Rocklon'	MAST (13101)
H. aspera 'NCSU'	MAST (72800)
H. aspera ssp. *robusta*	S. Reed (G629A)
H. aspera ssp. *sargentiana*	MAST (44900)
H. aspera ssp. *sargentiana* 'Sargent'	Bell Family Nursery
H. aspera ssp. *strigosa*	S. Reed (G630A)
H. aspera ssp. *villosa* 1	MAST (45100)
H. aspera ssp. *villosa* 2	MAST (45100)
H. aspera ssp. *villosa* 3	Bell Family Nursery
H. anomala ssp. *petiolaris* 1[y]	S. Reed (G132A)
H. anomala ssp. *petiolaris* 2	Bell Family Nursery
H. anomala ssp. *petiolaris* 'Mirranda'	MAST (3005)
H. anomala ssp. *petiolaris* group *quelpartensis* 1[y]	S. Reed (G436A)
H. anomala ssp. *petiolaris* group *quelpartensis* 2	Bell Family Nursery
Platycrater arguta[z]	S. Reed (G718B)
H. scandens ssp. *chinensis* group *angustipetala* 1	MAST (2905)
H. scandens ssp. *chinensis* group *angustipetala* 2	S. Reed (G627A)
H. scandens ssp. *chinensis* group *lobbii*	S. Reed (G520E)
H. scandens ssp. *liukiuensis* group *luteovenosa* 1[y]	MAST (46300)
H. scandens ssp. *liukiuensis* group *luteovenosa* 2	S. Reed (G525D)
H. scandens ssp. *liukiuensis* group *luteovenosa* 'Aureomarginata'	MAST (38998)
Schizophragma hydrangeoides[y]	Bell Family Nursery
Schizophragma hydrangeoides 'Moonlight'[y]	S. Reed (G719B)
H. heteromalla 1	S. Reed (G432A)
H. heteromalla 2	S. Reed (G639A)
H. heteromalla 3	S. Reed (G640A)
H. heteromalla group *xanthoneura*	S. Reed (G644A)
H. paniculata 'Brussel's Lace'	Bell Family Nursery
H. paniculata 'Greenspire'	MAST (65001)
H. paniculata 'Limelight'	S. Reed (G582C)
H. paniculata 'Pee Wee'	S. Reed (G652A)
H. paniculata 'Pink Diamond'	S. Reed (G653B)
H. paniculata 'Tardiva'	S. Reed (G654A)
H. macrophylla 'Alpengluhen'	MAST (43200)
H. macrophylla 'Ayesha'	Bell Family Nursery
H. macrophylla 'Blaumeise'	S. Reed (G598A)
H. macrophylla 'DavidRamsey'	MAST (37902)
H. macrophylla 'Dooley'	Bell Family Nursery
H. macrophylla 'Eisvogel'	S. Reed (G884)
H. macrophylla Endless Summer ('Bailmer')	Bell Family Nursery
H. macrophylla 'Holstein'	S. Reed (G344B)
H. macrophylla 'Izu No Hana'	MAST (44800)

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ABI3730xl using ROX-500 size standard (Applied Biosystems, Foster City, Calif.). GeneMapper version 3.7 was used to recognize and size peaks (Applied Biosystems).

Data analysis. Data from 14 SRR loci were compiled for 85 samples and analyzed for shared allele frequencies. Among population variation was calculated for each subsection and species by comparing effective numbers of alleles to differences in allele frequencies between taxa. Assignment tests were not carried out since populations were based on subsection and species designations. Gene diversity estimates were produced using Nei's 1987 estimator for heterozygosity and unbiased gene diversity per populations using FSTATS software (Goudet, 1995; Saitou and Nei, 1987). Allele sharing statistics were used independent of ploidy differences and all alleles were represented as diploid. Average gene diversity was calculated between populations (Dst) and calculated relative to total gene diversity (Gst) (Nei, 1973).

Populations version 1.2.28 was used for phenetic analyses (Langella, 2002). Genetic distances between individual samples were calculated using allele sharing distance (DAS) to create a distance matrix (Jin and Chakraborty, 1994; Stephens et al., 1992). Neighbor-joining with 100 bootstrap replicates for statistical support was used to generate tree phenograms showing clustering of genetically similar samples (Saitou and Nei, 1987). Phenograms were visualized with TreeView (Page, 1996).

Results

Marked species listed in Table 1 were processed with SSR primers described in Table 2. Nine of the 14 SSR loci share sequence similarity with known genes and proteins. All repeats are trinucleotide and all but three loci produced a range of actual allele sizes that included the predicted size. The number of alleles varied from 4 to 17. A complete list of the samples and their origin is shown in Table 3. Seven of the 85 samples failed to generate data with all 14 SSR primers (Table 3). *Schizophragma hydrangeoides* Sieb. and Zucc. is missing data for four loci, *S. hydrangeoides* 'Moonlight' for two loci and *P. arguta* for three loci. Individual samples for *H. anomala* D. Don, *H. scandens* (L.) Ser., and *H. macrophylla* ssp. *serrata* are missing data, but in these cases there are additional samples for each taxa that produced data for all 14 SSR loci.

Table 3. Continued.

Taxa	Source[z] (accession no.)
H. macrophylla 'Jogasaki'	MAST (47600)
H. macrophylla 'Kardinal'	S. Reed (G597B)
H. macrophylla 'Lemon Wave'	Bell Family Nursery
H. macrophylla 'Libelle White'	MAST (46400)
H. macrophylla 'Mariesii'	MAST (43101)
H. macrophylla 'Nightingale'	MAST (46500)
H. macrophylla 'Nigra'	S. Reed (G456B)
H. macrophylla 'Nikko Blue'	S. Reed (G596B)
H. macrophylla 'Penny Mac'	S. Reed (G683B)
H. macrophylla 'Pia'	Bell Family Nursery
H. macrophylla 'Seafoam'	Bell Family Nursery
H. macrophylla 'Veitchii'	Bell Family Nursery
Dichroa febrifuga	S. Reed (G717C)
Dichroa febrifuga 'Yamaguchi Select'	MAST (27004)
Dichroa versicolor 'Hogan'	MAST (43402)
H. macrophylla ssp. *serrata* 'Beni Gaku'	Bell Family Nursery
H. macrophylla ssp. *serrata* 'Bluebird'	MAST (26500)
H. macrophylla ssp. *serrata* group fertilis	MAST (8102)
H. macrophylla ssp. *serrata* group forma chinensis[y]	MAST (49700)
H. macrophylla ssp. *serrata* 'Miranda'	MAST (46900)
H. macrophylla ssp. *serrata* 'Omacha'	Bell Family Nursery
H. macrophylla ssp. *serrata* 'Shichidanka'	S. Reed (G705D)
H. macrophylla ssp. *serrata* 'Tiara'	MAST (47400)
H. macrophylla ssp. *serrata* 'Woodlander'	MAST (75299)
H. macrophylla ssp. *serrata* 'Yae No Amacha'	MAST (47500)
H. seemannii 1	S. Reed (G434A)
H. seemannii 2	Bell Family Nursery
H. integrifolia	S. Reed (G633A)
H. serratifolia	S. Reed (G567B)

[z]Bell Family Nursery = Bell Family Nursery, Inc., Aurora, Ore.; M. Dirr = M.A. Dirr, Shade Garden Hydrangea Collection, University of Georgia, Athens; MAST = MAST Arboretum, Steven F. Austin State Univ., Nacogdoches, Texas; S. Reed = S.M. Reed, USDA–ARS, McMinnville, Tenn.
[y]Indicates samples that failed to generate data for one or more of the 14 SSR loci tested.

Measures of genetic diversity varied considerably between loci. Heterozygosity (Ho) using Nei's (1987) estimation indicated that the STAB111_112 locus generated the most variation while STAB457_458 locus had the least (Table 4). STAB061_062, STAB125_126, STAB307_308, and STAB309_310 also had low genetic diversity indicating low numbers of polymorphic alleles for these loci (Table 4). The most diverse loci were STAB111_112 and STAB285_286 with the next most diverse locus being STAB311_312 with 10% less polymorphic alleles. The range of heterozygosity suggests that these SSR are appropriate for the subsection and species level comparisons.

Number of alleles per locus was calculated for all samples and for each subsection individually. As expected, STAB457_458 had the most fixed loci with six and the least number of total alleles (Table 4). Loci with low genetic diversity generally had fewer alleles and more allele fixation. However, higher genetic diversity did not necessarily mean more alleles since STAB311_312 had the most alleles at 17, including one fixed for *Macrophyllae*, but had less observed heterozygosity, or genetic diversity, than STAB111_112 and STAB285_286. Four loci did not produce fixed alleles for any subsection, but each subsection included at least three fixed loci. Data for *Dichroa*, *Platycrater*, and *Schizophragma* samples were not included in subsection analyses since their placement in the tribe has not been established.

Diversity within and between populations was calculated several ways. Total gene diversity (Ht) was calculated for each locus and the mean for all loci is 0.735 while the average gene diversity (Hs) for each locus is 0.370 (Table 4). If samples were randomly chosen from subsections they should differ on average at 37% of their loci. If they are chosen from the whole sample the difference increases to 73.5%. Dst and Gst values are 0.366 and 0.497, respectively. Thus, 49.7% of the overall variation is due to differences between subsections and diversity among subsections was 36.6%. Gene diversity measures were also calculated for populations defined by species labels (data not shown). When analyzed by species, Dst and Gst values are higher at 0.454 and 0.617, respectively. In other words, 61.7% of the total diversity is due to differences between species.

Genetic distance measures for each subsection ranged from 0.328 between *Calyptranthae* (Maxim.) E.M. McClint. and *Asperae*, indicating greater genetic similarity between these subsections, to 0.901 between *Macrophyllae* and section *Cornidia*, suggesting significant genetic divergence between these taxa (Table 5). Samples in subsection *Macrophyllae* appear genetically similar to *Petalanthe* and both subsections cluster separately from other taxa (Fig. 2).

Most of the associations between subsections remain intact when samples are analyzed at the species level. Data include *Platycrater*, *Schizophragma*, and *Dichroa* genera. Figure 3 shows *H. macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* are genetically similar to each other and to *Dichroa* and *H. scandens*. All four species are removed from other taxa (Fig. 3, 88% bootstrap support). *Hydrangea heteromalla* D. Don and *H. paniculata* of subsection *Heteromallae* cluster with 100% bootstrap. *Schizophragma* shares considerable genetic similarity with *H. heteromalla* and *H. paniculata*. Species did not always associate within their subsection. For example, *H. involucrata* and *H. aspera* of subsection *Asperae* are only associated with minimal bootstrap support and *H. involucrata* appears more closely related to *H. anomala* of subsection *Calyptranthae* than to *H. aspera* (54% bootstrap support). *Platycrater* shares genetic similarity with species in section *Cornidia* but bootstrap support is low at 71% (Fig. 3).

Genetic similarity between individual samples is shown in Fig. 4, which is rooted with *S. hydrangeoides* since SSR loci used here did not amplify with other outgroups. All samples cluster by species except for *H. macrophylla* cultivars Pia, Kardinal, and Veitchii, which share genetic similarity with *H. macrophylla* ssp. *serrata*. Grouping between samples is generally consistent with subsection- and species-level results shown in Figs. 2 and 3. *Hydrangea paniculata* and *H. heteromalla* cluster together with 87% bootstrap support and share genetic similarity with *Schizophragma* samples. As expected, *Dichroa*, *H. scandens*, and *H. macrophylla* cluster separate from other taxa. On the other hand, *H. arborescens* and *H. quercifolia* are genetically similar

Table 4. Gene diversity and number of alleles per locus for entire population and within *Hydrangea* subsections. Data does not include *Dichroa*, *Platycrater*, and *Schizophragma* plants. Zero values for gene diversity within subsections indicate homozygous, fixed loci within that subsection.

	Genetic diversity		Gene diversity within subsections (alleles per locus)										
Locus	Ho[z]	Alleles (no.)	Ht[y]	Hs[x]	Dst[w]	Gst[v]	*Macrophyllae*	*Petalanthe*	*Heteromallae*	*Americanae*	*Cornidia*	*Calyptranthae*	*Asperae*
STAB045_046	0.43914	10	0.651	0.018	0.633	0.972	0.742 (6)	0.783 (4)	0.361 (4)	0.455 (4)	0.667 (2)	0.500 (2)	0.753 (5)
STAB061_062	0.03325	6	0.584	0.033	0.551	0.943	0.151 (2)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.071 (2)
STAB111_112	0.76267	12	0.898	0.618	0.28	0.312	0.737 (6)	0.433 (3)	0.500 (2)	0.768 (4)	0.792 (4)	0.500 (2)	0.604 (4)
STAB125_126	0.09568	9	0.72	0.323	0.398	0.552	0.543 (5)	0.300 (2)	0.533 (2)	0.536 (2)	0.000 (1)	0.000 (1)	0.258 (2)
STAB157_158	0.34388	11	0.853	0.516	0.337	0.395	0.000 (1)	0.833 (5)	0.756 (4)	0.688 (3)	0.000 (1)	0.450 (2)	0.846 (7)
STAB285_286	0.71626	10	0.789	0.532	0.258	0.327	0.494 (2)	0.583 (3)	0.783 (5)	0.518 (2)	0.250 (2)	0.500 (2)	0.571 (3)
STAB307_308	0.09491	9	0.588	0.193	0.395	0.672	0.491 (5)	0.167 (2)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.618 (4)
STAB309_310	0.07558	8	0.767	0.349	0.418	0.545	0.359 (2)	0.000 (1)	0.689 (3)	0.750 (3)	0.667 (2)	0.000 (1)	0.000 (1)
STAB311_312	0.60918	17	0.879	0.577	0.301	0.343	0.000 (1)	0.500 (3)	0.906 (11)	0.750 (4)	0.833 (5)	0.350 (2)	0.712 (4)
STAB321_322	0.16820	9	0.483	0.17	0.313	0.648	0.746 (6)	0.000 (1)	0.000 (1)	0.393 (2)	0.000 (1)	0.000 (1)	0.000 (1)
STAB391_392	0.28840	7	0.662	0.302	0.36	0.544	0.532 (3)	0.500 (2)	0.000 (1)	0.598 (4)	0.000 (1)	0.000 (1)	0.440 (2)
STAB429_430	0.47132	8	0.769	0.358	0.411	0.534	0.567 (4)	0.300 (2)	0.189 (2)	0.000 (1)	0.500 (2)	0.450 (2)	0.467 (2)
STAB457_458	0.01786	4	0.791	0.608	0.182	0.231	0.000 (1)	0.000 (1)	0.000 (1)	0.125 (2)	0.000 (1)	0.000 (1)	0.000 (1)
STAB501_502	0.51343	13	0.859	0.578	0.281	0.327	0.567 (5)	0.700 (3)	0.194 (3)	0.536 (2)	0.833 (4)	0.500 (2)	0.687 (4)
Mean			0.735	0.370	0.366	0.497							

[z]Ho = observed heterozygosity, or genetic diversity, for all samples.
[y]Ht = total gene diversity.
[x]Hs = average gene diversity within subsections.
[w]Dst = average gene diversity between subsections.
[v]Gst = genetic differentiation between subsections.

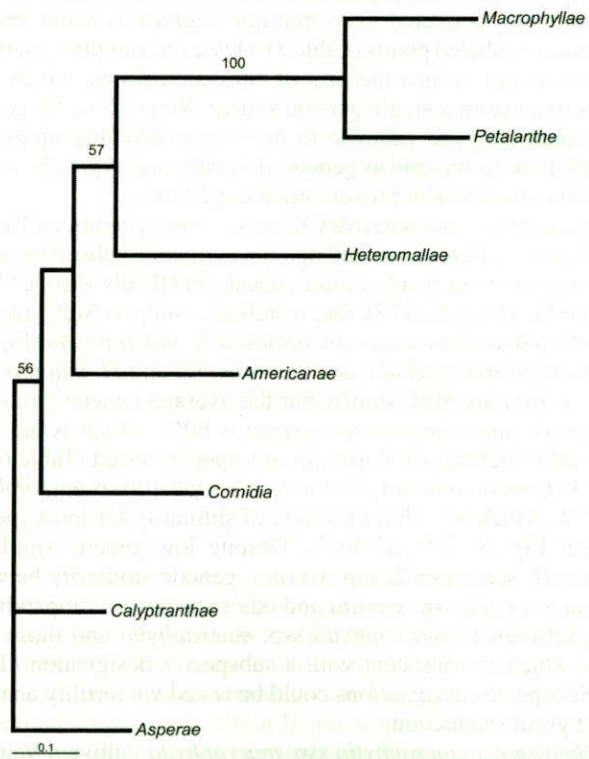

Fig. 2. This unrooted, neighbor-joining tree is based on allele sharing distances calculated for populations and indicates relationships, or clusters, between subsections. Data did not include *Dichroa*, *Platycrater*, and *Schizophragma* genera. Numbers correspond to 100 bootstrap replicates where higher numbers indicate increased statistical support. Bootstrap values less than 50 are not shown.

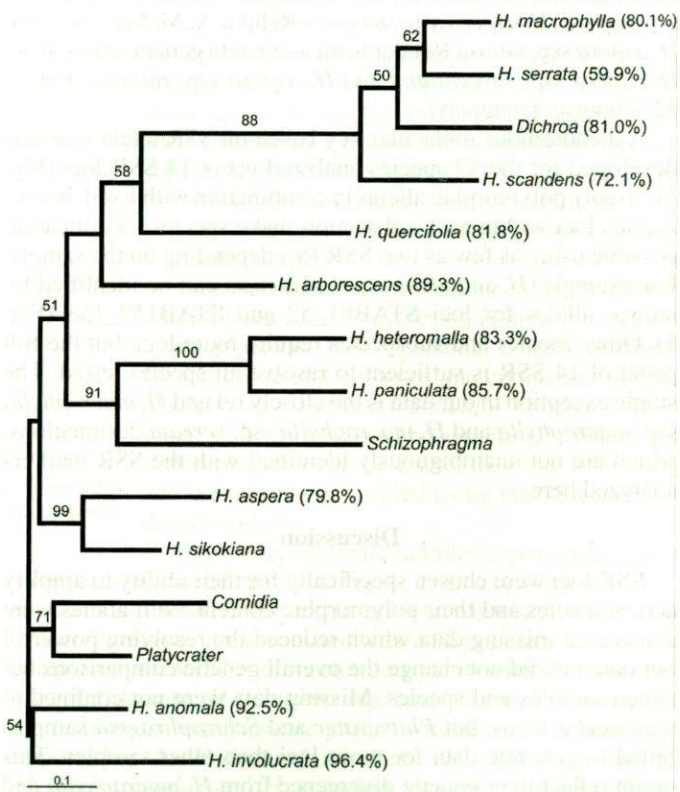

Fig. 3. Neighbor-joining tree was created using allele sharing distance and analysis of populations based on species groups. 100 bootstrap replicates were used for statistical support and percentages are shown above branches. Average genetic similarity within each species is shown next to each species except for species only represented by two or less samples. Bootstrap values less than 50 are not shown.

Table 5. Allele sharing distances between *Hydrangea* subsections. Higher values indicate greater genetic distance between taxa and lower values suggest increased genetic similarity. Data for *Dichroa*, *Platycrater*, and *Schizophragma* samples were not included since their placement within *Hydrangea* subsections is not well defined.

	Macrophyllae	*Petalanthe*	*Heteromallae*	*Americanae*	*Cornidia*	*Calyptranthae*	*Asperae*
Macrophyllae	0						
Petalanthe	0.384950	0					
Heteromallae	0.887048	0.862039	0				
Americanae	0.802261	0.800027	0.694377	0			
Cornidia	0.901107	0.881984	0.683460	0.540729	0		
Calyptranthae	0.862428	0.855892	0.636332	0.473218	0.411499	0	
Asperae	0.886986	0.885090	0.627525	0.519281	0.483813	0.328404	0

but are included in a large group that includes section *Cornidia* and subsection *Asperae* samples. Genetic similarity between species is relatively uninformative when compared to Fig. 3. For example, *P. arguta* and *H. sikokiana* appear genetically similar to *H. anomala* instead of *H. aspera* and Section *Cornidia*.

Associations between subspecies and individual samples are better supported by bootstrap analysis in Fig. 4. *Hydrangea macrophylla* cultivars Dooley, Nikko Blue, Penny Mac, David Ramsey, and Bailmer (Endless Summer) are remontant and were previously described using RAPD markers (Lindstrom et al., 2003). Their genetic similarity is confirmed here (Fig. 4, 82% and 81% bootstrap support). *Hydrangea scandens* ssp. *chinensis* (Maxim.) E.M. McClint. and *H. scandens* ssp. *liukiuensis* (Nakai) E.M. McClint. cluster separately. *Hydrangea anomala* D. Don ssp. *petiolaris* (Sieb. and Zucc) E.M. McClint. samples that were sold under the name "*H. quelpartensis*" appear genetically distinct from other *H. anomala* ssp. *petiolaris* samples. *Hydrangea aspera* ssp. *aspera*, *H. aspera* ssp. *strigosa* (Rehder) E.M. McClint., and *H. aspera* ssp. *villosa* Rehder form a separate genetic group from *H. aspera* ssp. *sargentiana* and *H. aspera* ssp. *robusta* (Fig. 4, 62% bootstrap support).

A dichotomous molecular key based on SSR allele size was developed for the 17 species analyzed using 14 SSR loci (Fig. 5). Highly polymorphic alleles in combination with fixed, homozygous loci within each subsection make species identification possible using as few as two SSR loci depending on the sample. For example, *H. scandens* ssp. *liukiuensis* can be identified by unique alleles for loci STAB61_62 and STAB157_158 (Fig. 5). Other species and subspecies require more loci, but the full panel of 14 SSR is sufficient to resolve all species tested. The single exception in our data is the closely related *H. macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* designations, which are not unambiguously identified with the SSR markers analyzed here.

Discussion

SSR loci were chosen specifically for their ability to amplify across species and their polymorphic content. Null alleles were considered missing data which reduced the resolving power of our data but did not change the overall genetic comparisons between samples and species. Missing data were not confined to a particular locus, but *Platycrater* and *Schizophragma* samples failed to generate data for more loci than other samples. This might reflect their genetic divergence from *H. macrophylla* and *H. paniculata*, the DNAs used to construct the SSR-enriched library. Nine of the microsatellite regions share sequence similarity with known genes. Several are genes coding for conserved plant functions which is expected since these loci amplify in 17 different species (Rossetto, 2001).

We tested several genetic distance methods including step-wise mutation models for SSR evolution such as ASD, D_{sw}, and DMU2 (Goldstein and Clark, 1995; Goldstein and Pollock, 1997; Pollock et al., 1998; Shriver et al., 1995). Empirical testing of the step-wise mutation models resulted in unstable phylogenies, probably because our SSR loci are highly polymorphic and allele sizes varied greatly (Table 2). We did not sequence repeats in all species to confirm that allele size variation only represents differences in repeat number and does not include additional mutations and imperfections. Proper use of step-wise mutation models would require that we constrain allele size ranges and estimate mutation rates. The underlying purpose of this work was to enhance hydrangea breeding by establishing a molecular key for *Hydrangea* species which is better accomplished when the allele size range is not constrained. Accordingly, most of our samples are named cultivars and do not necessarily represent genetic diversity found in wild populations. Samples were duplicated and intentionally collected from multiple sources to better protect against mislabeled plants (Table 3). However, sampling methods did not guard against the bias of cultivated plants, which may be derived from a single genetic source. Regardless, the results presented here are valuable to hydrangea breeding programs, which look to maximize genetic diversity and typically utilize named cultivars with proven ornamental traits.

According to our data, McClintock correctly removed *Macrophyllae* from *Petalanthe* and made it a separate subsection since the species in each subsection appear genetically distinct from each other (Figs. 2 and 3). Our results also support McClintock's assertion that *serrata* is a subspecies of *H. macrophylla* (Fig. 4). *Hydrangea macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* are 80% similar but the average genetic similarity within *H. macrophylla* ssp. *serrata* is 60%, which is the least amount of genetic similarity for any species tested (Table 6 and Fig. 3). Genetic similarity within *H. macrophylla* ssp. *macrophylla* is 80%, which is within the range of similarity for most species tested (Fig. 3, 72% to 96%). Despite low genetic similarity within *H. macrophylla* ssp. *serrata*, genetic similarity between *H. macrophylla* ssp. *serrata* and other species is comparable to that between *H. macrophylla* ssp. *macrophylla* and those species, which is consistent with a subspecies designation (Table 6). Subspecies designations could be tested via fertility analysis and hybrid production.

Hydrangea macrophylla ssp. *macrophylla* cultivars Veitchii, Pia, and Kardinal appear more genetically similar to *H. macrophylla* ssp. *serrata* than to other *H. macrophylla* ssp. *macrophylla* cultivars (Fig. 6). Their placement between subspecies groups suggests that they may be hybrids. The hybrid nature of a few *H. macrophylla* ssp. *serrata* cultivars was proposed by Zonneveld (2004) after uncovering genome sizes that were intermediate

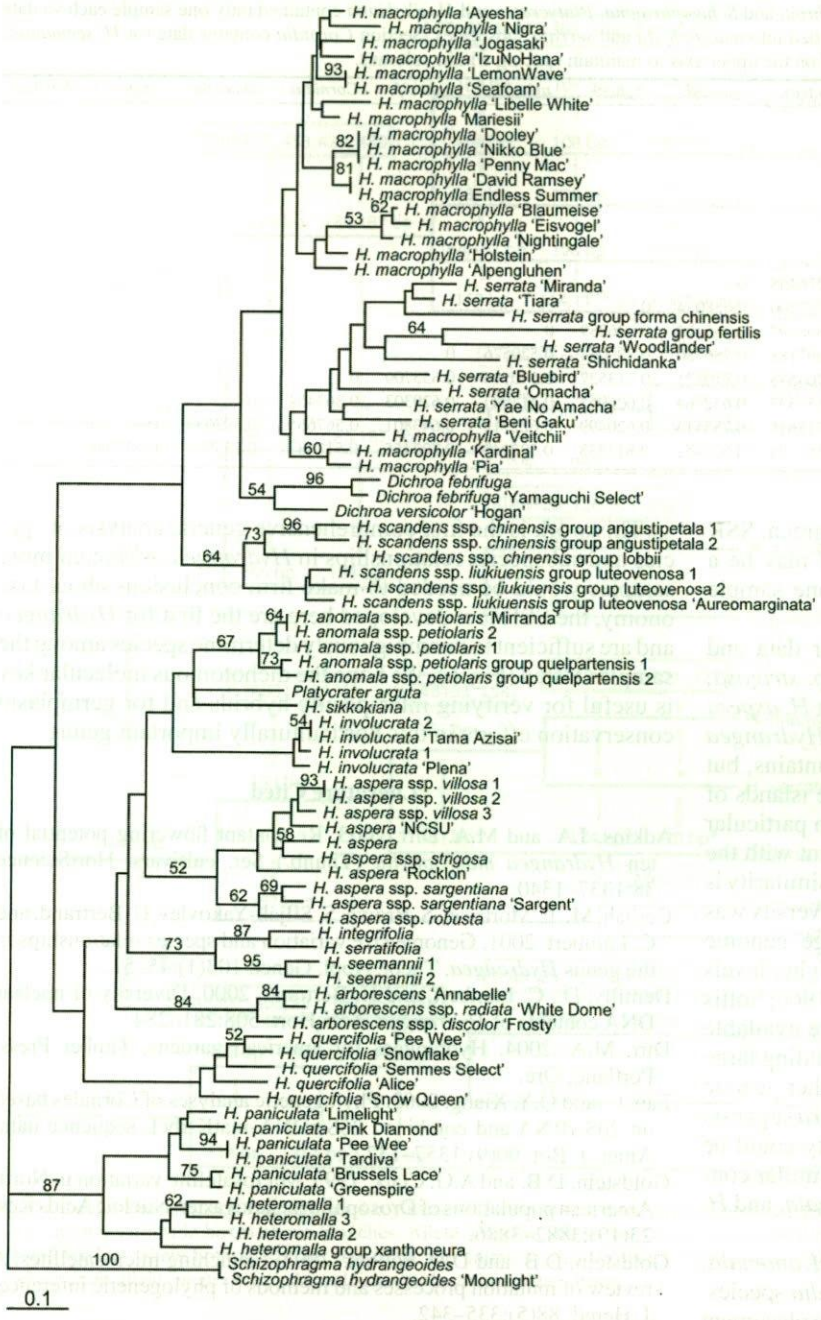

Fig. 4. This neighbor-joined tree includes all individual data used in our analysis and is rooted with Schizophragma samples. Numbers correspond to 100 bootstrap replicates where higher numbers indicate more statistical support. Bootstrap values less than 50 are not shown.

between *H. macrophylla* ssp. *serrata* and *H. macrophylla* ssp. *macrophylla*, which the author suggests are two separate species. The detection of probable hybrids, a lack of unique SSR alleles in our molecular key, and the close genetic distance support the subspecies designations introduced by McClintock (1957).

Several known links within *H. macrophylla* ssp. *macrophylla* cultivars are confirmed by our analyses. 'Dooley', 'Nikko Blue', 'David Ramsey', and 'Endless Summer' share remontant flowering traits and the SSR-derived phylogeny matches previous RAPD analysis results (Adkins and Dirr, 2003; Lindstrom et al., 2003). 'Seafoam' and 'Lemon Wave' are both variegated and appear joined with 93% bootstrap support (Fig. 6). 'Blaumeise', 'Eisvogel', and 'Nachtigall' are known triploids (Zonneveld, 2004) produced by the same breeding program and cluster with 53% bootstrap support. A more detailed study of *H. macrophylla* ssp. *macrophylla* and *H. macrophylla* ssp. *serrata* is under way with a larger pool of SSR markers.

Macrophyllae and *Petalanthe* appear to be more closely related to each other than to the rest of the *Hydrangea* species (Table 5; Figs. 2 and 3). This divergence is consistent with the consensus tree for *Hydrangea* in Fig. 1 but has consequences for *Hydrangea* breeding since the most popular and colorful hydrangeas are *H. macrophylla* cultivars. Introgressing flower color and other ornamental traits through interspecific breeding has proven to be difficult (Kudo and Niimi, 1999; Reed, 2000, 2004; Reed et al., 2001). According to SSR data, the exceptions should be *H. scandens* and *Dichroa* species which are tightly grouped with *H. macrophylla* (Fig. 5) and share a considerable number of alleles (Table 6). The relatively high coefficient of differentiation (Gst) suggest geographic or ecological isolation of the subsections (Table 4). Within and between subspecies diversity, Hs and Dst, were approximately equal and genetic similarity clustering shows clear distinction between subsections. Results for species indicate that the average gene diversity within species was lower (0.282) and the average diversity between species was higher (0.454). The resulting higher Gst value (0.617) suggests that more of the total diversity is due to differentiation between species. These statistics should be regarded as a convenient summary since there are a relatively small number of loci involved and diversity and distance for the entire genome would require more loci. Comparable results between subsection and species-level analyses is most likely due to the fact that the seven subsections are only subdivided into 11 species groups with three of the groups retaining the same samples.

Genetic distance alone is a poor indicator of compatibility since *Dichroa febrifuga* Lour. is potentially a hexaploid (K. Jones, personal communication). Genome size and chromosome counts are not yet available for *Dichroa versicolor* (Fortune) D.R. Hunt. Fertility needs to be empirically tested but if wide hybrids can be made, they can be rapidly and inexpensively verified using the SSR markers described here.

Hydrangea anomala samples appear to segregate into two groups based on origin (Fig. 4). *Hydrangea anomala* ssp. *petiolaris* is generally collected from Japan while samples labeled "*H. quelpartensis*" are associated with wild populations in South Korea. Both are aerial-rooting climbing plants with white lacecap flowers. Likewise, *H. scandens* ssp. *chinensis* and *H. scandens* ssp. *liukiuensis* form separate groups with reasonable bootstrap support (Fig. 4). *Hydrangea scandens* ssp. *chinesis* is sold as "*H. angustipetala*" and *H. scandens* ssp. *liukiuensis* is sold as "*H. luteovenosa*." They are extant to Taiwan and the mountains of Japan, respectively. *Hydrangea scandens* ssp. *chinesis* group *angustipetala* are deciduous while *H. scandens* ssp. *liukiuensis* group *luteovenosa* plants are generally semi-evergreen. Interestingly, *H. scandens* ssp. *chinesis*, also sold as "*H. lobbii*," is found

Table 6. Allele sharing distances between *Hydrangea* species, *Dichroa*, and *Schizophragma*. *Platycrater* and *H. sikokiana* contained only one sample each so data were not included. *Hydrangea macrophylla* samples were divided into *macrophylla* and *serrata* subspecies. Section *Cornidia* contains data for *H. seemannii*, *H. integrifolia*, and *H. serratifolia*. Taxa labels are abbreviated on the upper axis to maintain spacing within columns.

	mac.	*serrata*	*Dichroa*	*scanden*	*hetero.*	*panicul.*	*Schizo.*	*arbores.*	*quercif.*	*Cornidia*	*anomala*	*aspera*	*Involucr.*
H. macrophylla ssp. *macrophylla*	0												
H. macrophylla ssp. *serrata*	0.193389	0											
Dichroa	0.249481	0.350568	0										
H. scandens	0.421015	0.385957	0.343507	0									
H. heteromalla	0.895931	0.867574	0.952899	0.860489	0								
H. paniculata	0.899819	0.929760	0.907143	0.881215	0.276408	0							
Schizophragma	0.895772	0.916559	0.966228	0.888415	0.737500	0.638034	0						
H. arborescens	0.858754	0.918000	0.878788	0.908059	0.806897	0.755102	0.806289	0					
H. quercifolia	0.824333	0.801392	0.865399	0.782513	0.697188	0.786780	0.889064	0.529576	0				
Cornidia	0.906067	0.903134	0.961538	0.881984	0.704545	0.710821	0.713527	0.540146	0.655700	0			
H. anomala	0.868175	0.837373	0.904551	0.843299	0.657337	0.632964	0.606963	0.417587	0.629303	0.363454	0		
H. aspera	0.908121	0.917714	0.940770	0.894446	0.717656	0.755513	0.620499	0.622712	0.643801	0.567665	0.372634	0	
H. involucrata	0.897871	0.891669	0.932886	0.914130	0.587748	0.627451	0.813333	0.592949	0.727455	0.517483	0.355913	0.542098	0

in the Philippines and Taiwan and listed as semi-evergreen. SSR data suggest *H. scandens* ssp. *chinesis* group *lobbii* may be a hybrid between *H. scandens* subspecies but only one sample has been tested.

Five *H. aspera* subspecies were included in our data and results indicate *H. aspera* ssp. *aspera*, *H. aspera* ssp. *strigosa*, and *H. aspera* ssp. *villosa* form a separate group from *H. aspera* ssp. *sargentiana* and *H. aspera* ssp. *robusta* (Fig. 4). *Hydrangea aspera* occur in China, mainly in the Himalaya Mountains, but also in western and south central regions, and on the islands of Formosa, Sumatra, and Java (McClintock, 1957). No particular geographic or morphological information is consistent with the groups shown in Fig. 4 and the within species genetic similarity is comparable to other species (Fig. 3). Greater genetic diversity was expected because previous reports of geographic range, genome size ranges, and morphological variation indicated higher levels than other *Hydrangea* species (Table 1) (Hutchinson, 1967; Soltis et al., 1995; Zonneveld, 2004). Several cultivars are available and plants such as 'Sargent' have desirable traits including large fuzzy leaves and deep purple flowers suggesting further genetic testing is warranted. Sample clustering within *H. aspera* suggests genetic structure within species and genetic diversity could be maximized when breeding by selection of parents. Similar conclusions could be made for *H. macrophylla*, *H. anomala*, and *H. paniculata* (Fig. 4).

Despite the genetic similarity between *H. aspera*, *H. anomala*, *H. involucrata*, *H. sikokiana*, *Platycrater*, and *Cornidia* species, the relationships between these species, particularly the placement of *P. arguta*, is not well resolved. Bootstrap support at these nodes is low suggesting that Fig. 4 may not be accurate. Likewise, our SSR results do not contradict the consensus tree placement of *H. quercifolia* and *H. arborescens*, which are shown separate (Fig. 1). While a unified subsection *Americanae* is not necessarily ruled out, there is little support among the genetic data for a relationship such as that seen for *H. heteromalla* and *H. paniculata* of subsection *Heteromallae* (Figs. 3 and 4).

On the other hand, *Schizophragma* consistently appears joined to subsection *Heteromallae* including as much as 95% bootstrap support at the species level (Fig. 4). The *Schizophragma* clade in Fig. 1 contains *Decumaria* and *Pileostegia*, and is shown associated with *H. quercifolia* of subsection *Americanae*. Our results suggest the *Schizophragma* clade is genetically similar to subsection *Heteromallae*, although we cannot specifically address *Decumaria* and *Pileostegia* genera.

This work is the first comprehensive genetic analysis of species and subspecies relationships in *Hydrangea*. Although more samples would be needed to make firm conclusions about taxonomy, the SSR loci developed here are the first for *Hydrangea* and are sufficient to unambiguously determine species among the samples tested (Fig. 6). We hope the dichotomous molecular key is useful for verifying interspecific hybrids and for germplasm conservation efforts in this horticulturally important genus.

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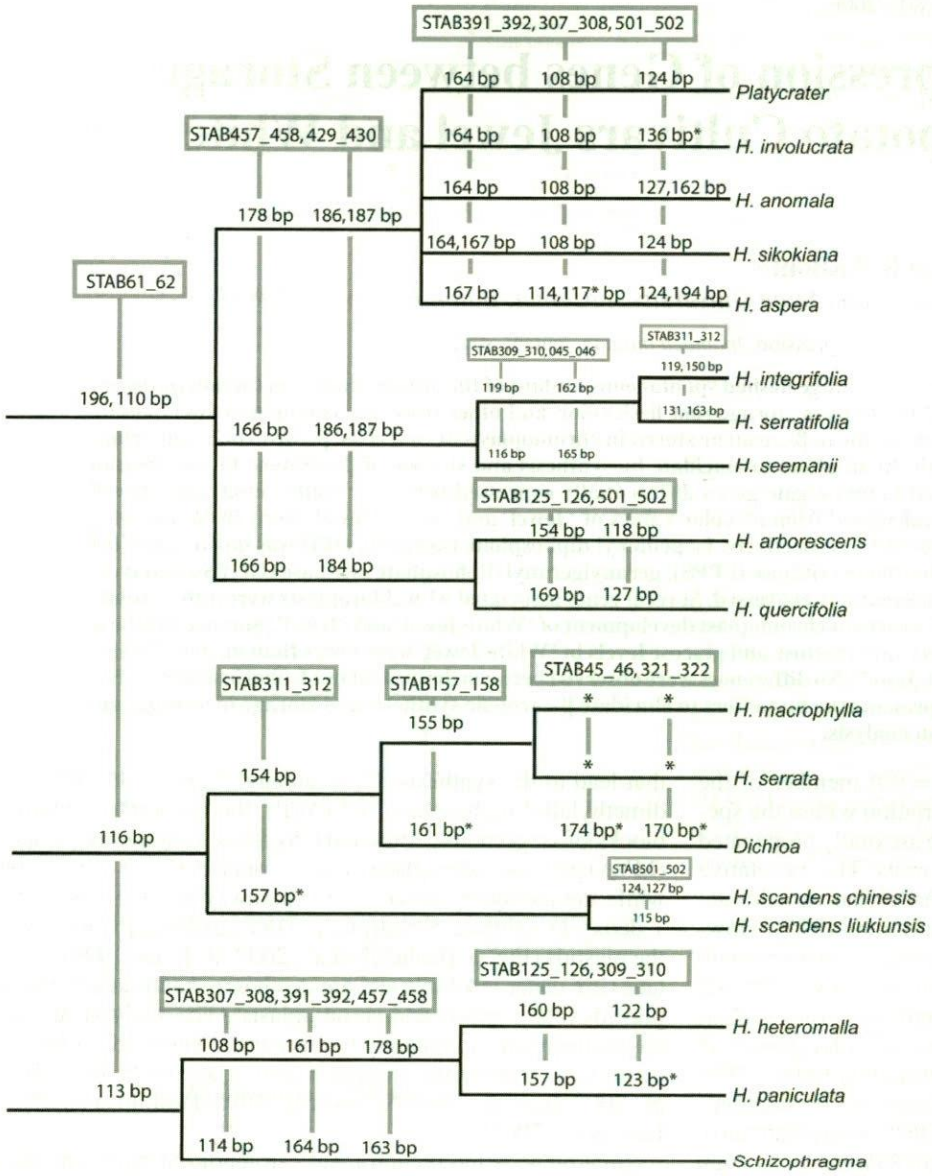

Fig. 5. Species and subspecies dichotomous key using 14 SSR markers is shown as a modified dendogram. SSR loci are named inside boxes above branches. Allele sizes are shown below each locus. Multiple alleles are listed for loci where both alleles are consistent and unique to that taxonomic group. Asterisks indicate loci where extra alleles were observed in addition to the unique sizes shown.

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